

# The Region Encoded by the Alternatively Spliced Exon IIIA in Mesenchymal Fibronectin Appears Essential for Chondrogenesis at the Level of Cellular Condensation

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Fibronectin in the extracellular matrix of tissues acts as a substrate for cell adhesion and migration during development. Heterogeneity in the structure of fibronectin is largely due to the alternative splicing of at least three exons (IIIB, IIIA, and V) during processing of a single primary transcript. Fibronectin mRNA alternative splicing patterns change from B+A+V+ to B+A–V+ during chondrogenesis. In this report, immunohistochemical analysis demonstrates that while fibronectin protein containing the region encoded by exon IIIB is present throughout the limb at all stages of development, fibronectin protein containing the region encoded by exon IIIA disappears from cartilaginous regions just after condensation *in vivo* and in high-density mesenchymal micromass cultures *in vitro*. Treatment of mesenchymal micromass cultures prior to condensation with an antibody specific for the region encoded by exon IIIA disrupts the formation of cellular condensations and inhibits subsequent chondrogenesis in a dose- and time-dependent manner. Furthermore, microinjection of the exon IIIA antibody into embryonic chick limb primordia *in vivo* results in malformations characterized by smaller limbs and loss of limb skeletal elements. These results strongly suggest that the presence of the region encoded by exon IIIA in mesenchymal fibronectin is necessary for the condensation event that occurs during chondrogenesis. © 1997 Academic Press

## INTRODUCTION

Fibronectin (FN),<sup>2</sup> a large glycoprotein present in plasma and the extracellular matrix of most tissues, participates in many critical cellular processes including differentiation, adhesion, clot formation, and migration. Fibronectin generally exists in the extracellular matrix as a dimer of similar or identical subunits. Each fibronectin monomer is composed of three types of homologous repeating units, the type I, II, and III elements, that combine to form a series

of functional domains with specific binding affinities for structures such as the cell surface, collagen, fibrin, and heparin (for reviews see Hynes, 1990; Mosher, 1989). Even though the general structure of the fibronectin monomer is highly conserved, several different forms of fibronectin exist including a form present in plasma and multiple cell-associated forms (for reviews see Hynes, 1990; Mosher, 1989; Yamada, 1983). Some of the forms differ simply in the extent or type of glycosylation (Paul and Hynes, 1984). However, much of the heterogeneity in fibronectin's structure is due to the alternative splicing of at least three exons (IIIB, IIIA, and V) during the posttranscriptional processing of the fibronectin primary transcript (Schwarzbauer *et al.*, 1983, 1987b; Kornblihtt *et al.*, 1984; Odermatt *et al.*, 1985; Zardi *et al.*, 1987). Two of the alternatively spliced exons (IIIB and IIIA) can be either entirely present or entirely absent in the mature fibronectin mRNA as a result of exon skipping. Multiple splice variations involving complex exon subdivision exist for the third alternatively spliced exon containing the V (variable) region with the number of possible variations depending on the species. In the chick,

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<sup>2</sup> Abbreviations used: CMFSG, Ca<sup>2+</sup>, Mg<sup>2+</sup>-free saline plus glucose; FBS, fetal bovine serum; FN, fibronectin; PBS, phosphate-buffered saline; PNA, peanut agglutinin; RGD, arginine-glycine-aspartate; FGF, fibroblast growth factor; HH stage, Hamburger and Hamilton chick embryonic stage; MBP, maltose binding protein; N-CAM, neural cell adhesion molecule.

the entire V region may be included or the first 132 bases may be excluded.

Developmental and tissue-specific patterns of splicing have been demonstrated with each of the alternatively spliced regions of the fibronectin gene in many tissue and morphogenic processes (Norton and Hynes, 1987; French-Constant *et al.*, 1988, 1989; Pagani *et al.*, 1991; Magnuson *et al.*, 1991; Bennett *et al.*, 1991; DeSimone *et al.*, 1992). Furthermore, the expression of these alternatively spliced fibronectin mRNAs generally correlates with the expression of particular fibronectin isoforms (Price and Hynes, 1985; Paul *et al.*, 1986; Schwarzbauer *et al.*, 1987a). These observations support the idea that the splicing process provides a means to create different forms of fibronectin in the extracellular matrix, presumably for functional diversity. For example, fibronectin containing the region encoded by exon IIIA increases during the activation of lipocytes into contractile myoepithelial cells in an *in vitro* liver fibrosis model and appears to be required for the wound-healing process in this system (Jarnagin *et al.*, 1994). However, the functional significance of fibronectin isoform changes in most differentiation or wound-healing processes has not yet been established (French-Constant, 1995; Kornblihtt *et al.*, 1996).

One of these morphogenic processes, chondrogenesis, exhibits remarkable tissue and developmental specificity with respect to the alternative splicing of fibronectin mRNAs. Cartilage differentiation from mesenchymal tissues is characterized by specific changes in the extracellular matrix components present in each of these tissues. Mesenchyme contains a matrix rich in collagen type I and various types of heparan sulfate proteoglycans, while the newly formed cartilage matrix is composed primarily of collagen types II, IX, and XI and aggrecan, a large chondroitin sulfate proteoglycan (reviewed in von der Mark, 1980; Mayne, 1989; Kuettner, 1992). Fibronectin, a connecting molecule in the extracellular matrix, is present throughout chondrogenesis. However, we and others have shown that the structure of fibronectin changes during chondrogenesis as a result of specific alternative splicing events (Bennett *et al.*, 1991; White *et al.*, 1996; Peters *et al.*, 1996a,b). Specifically, all of the fibronectin mRNAs from prechondrogenic limb mesenchyme contains exons IIIB, IIIA, and V (B+A+V+), whereas all of the fibronectin mRNAs from chick cartilage contain exons IIIB and V but do not contain exon IIIA (B+A-V+). Furthermore, *in situ* hybridization with exon-specific probes reveals that the fibronectin mRNA splicing patterns change just after the condensation event that occurs during chick limb chondrogenesis both *in vivo* and *in vitro* (Gehris *et al.*, 1996).

In this report, we demonstrate that the changes in fibronectin alternative splicing that occur at the mRNA level (Bennett *et al.*, 1991; Gehris *et al.*, 1996) correspond to changes in fibronectin isoform expression at the level of the protein during chondrogenesis *in vivo* and *in vitro*. We show further that the addition of antibody or peptide specific for the region encoded by exon IIIA to high-density mesenchymal micromass cultures *in vitro* prior to condensation specifically disrupts cellular condensations and inhibits subse-

quent chondrogenesis. Injection of the exon IIIA antibodies into the limb primordia of shell-less chick embryos *in vivo* results in limb malformations characterized by smaller limbs and the absence of one or more skeletal elements. Taken together, these results suggest that the region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin is necessary for chondrogenesis, probably at the level of cellular condensation.

## MATERIALS AND METHODS

### Chick Embryos

Fertilized White Leghorn chicken eggs were obtained from Truslow Farms (Chestertown, MD) and incubated at 37.5°C in a humidified egg incubator for the desired period of time.

### Fibronectin Antibodies

Three anti-fibronectin antibodies were used as primary antibodies for the characterization of fibronectin isoforms in early and late stage chick limbs: (A) rabbit polyclonal antibodies raised against human plasma fibronectin from Cappel (Organon Teknika, West Chester, PA) that recognize all fibronectin isoforms, (B) a mouse monoclonal antibody specific for the EDA domain of human cellular fibronectin from ICN Biomedicals (Irvine, CA) that recognizes only the fibronectin isoforms containing the regions encoded by exon IIIA (Vartio *et al.*, 1987), and (C) rabbit polyclonal antibodies raised against the region encoded by exon IIIB in the chick fibronectin gene (White *et al.*, 1996). These antibodies will hereafter be referred to as the general fibronectin antibodies, the exon IIIA antibody, and the exon IIIB antibodies, respectively. We have shown previously that even though the general fibronectin antibodies and the exon IIIA antibody were raised against human fibronectins, these antibodies cross-react with the appropriate fibronectin isoforms in the chick (White *et al.*, 1996).

### Immunohistochemical Localization of the Alternatively Spliced Forms of Fibronectin

The distribution of fibronectin isoforms in paraformaldehyde-fixed, paraffin-embedded sections of chick limb buds at various stages of development was determined by immunohistochemical staining using a modification of the streptavidin-biotin system of the Histostain-SP kit (Zymed Laboratories Inc., South San Francisco, CA). The Histostain-SP method uses a biotinylated second antibody, a streptavidin-peroxidase conjugate, and a substrate-chromagen mixture to demonstrate the antigen in the cells and tissue. Specifically, early, middle, and late limb buds from Hamilton and Hamburger (HH) stages 20, 24, and 30 (Hamilton and Hamilton, 1951) were removed, rinsed in Dulbecco's phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde. The tissue was dehydrated in serial dilutions of ethanol and embedded in paraffin. Serial sections, 8 µm thick, were cut and mounted on Fisher plus slides, allowed to dry overnight on a 37°C slide warmer, and stored dry at 4°C until use. The slides were deparaffinized and washed in 0.1% acetic acid in PBS overnight. The tissue was then treated for 40 min with 300 U/ml hyaluronidase (bovine testes, Worthington Biochemical Co., Freehold, NJ) in 0.1 M sodium acetate buffer, pH 5.5, rinsed in PBS, and blocked with hydrogen perox-

ide in methanol. The method outlined in the kit was then utilized with a 1:100 dilution of each of the primary antibodies (see antibody section). The secondary antibodies were either anti-mouse or anti-rabbit from the kit. Color development was terminated in water and the slides were mounted in crystal mount and baked at 80°C for 30 min. The slides were viewed on an Olympus BH-2 microscope. Preimmune serum for the exon IIIB antibodies, mouse IgG, or rabbit serum replaced each of the primary antibodies in sections utilized as background controls. Staining with a monoclonal antibody specific for chick collagen type II (Linsenmayer and Hendricks, 1980; a generous gift of T. Linsenmayer, Tufts University) served as a marker for differentiated cartilage *in vivo* and cartilaginous nodules *in vitro*.

The distribution of fibronectin isoforms was also determined in whole-mount paraformaldehyde-fixed high-density mesenchymal micromass cultures (see next section) at 24, 48, and 72 hr after initial cell plating using the Histostain-SP kit as described above for tissue sections.

For immunofluorometric colocalization experiments, mesenchymal cells were initially plated in micromass droplets at a lower cell density ( $1 \times 10^6$  cells/ml) to facilitate subsequent visualization by fluorescence. In these experiments, rhodamine-conjugated anti-mouse secondary antibodies (for the exon IIIA antibody) and fluorescein-conjugated anti-rabbit secondary antibodies (for the exon IIIB antibodies), diluted 1:500 in PBS, were simultaneously incubated in the same sample for 10 min in order to visualize the positions of fibronectin isoforms containing the regions encoded by exon IIIA and exon IIIB in the same micromass culture. The samples for these experiments were viewed on an Olympus BH-2 microscope equipped with epifluorescence optics.

### High-Density Mesenchymal Micromass Cultures

Micromass culture is a modification of the system described by Ahrens *et al.* (1977) and San Antonio and Tuan (1986). Limb buds from HH stage 23/24 chick embryos were removed, cut in half, and placed in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free saline plus glucose (CMFSG) at 37°C. The limb buds were enzymatically dissociated in trypsin/collagenase in CMFSG containing 10% chick serum at 37°C for 45–60 min. The digestion process was terminated with the addition of 5% calf serum and the mixture was vortexed to disperse the cells. The cell suspension was then passed through a Nitex filter and the cells were pelleted by centrifugation. Cells were resuspended in CMFSG containing 5% calf serum, counted, and plated in 10- $\mu\text{l}$  drops at 10 or  $20 \times 10^6$  cells/ml. The cells were allowed to attach to 12-mm coverslips (Fisher) sitting in 24-well culture dishes for 2 hr at 37°C and 5%  $\text{CO}_2$  and then covered with 1 ml Ham's F12 media containing 10% FBS and penicillin (50 U/ml)/streptomycin (50 U/ml). Cultures were assessed 24, 48, and 72 hr after plating for chondrogenesis by staining with Alcian blue 8-GX (Sigma) at pH 1.0 as described (San Antonio and Tuan, 1986) and the expression of collagen type II mRNA by whole-mount *in situ* hybridization as described previously (Gehris *et al.*, 1996). Cultures were then viewed and photographed using an Olympus IMT-2 microscope equipped with Hoffman optics.

### Antibody Inhibition of Chondrogenesis in Vitro

Mesenchymal cells isolated from the limb buds of HH stage 23/24 chick embryos were plated as described above for high-density mesenchymal micromass cultures. Exon IIIA antibody (in concentrations ranging from 0.1 to 1.0  $\mu\text{g}/\text{ml}$ ) in Ham's F12 containing

10% FBS was added to the cultures 2 hr after cell plating. Additional antibodies used in inhibition studies included a mouse IgG control (Sigma); an avian-specific anti-fibronectin monoclonal antibody, B3D6 (Gardner and Fambrough, 1983), with an unmapped epitope, obtained from the Developmental Studies Hybridoma Bank (University of Iowa); the 16G3 monoclonal antibody (Nagai *et al.*, 1991) specific for an epitope mapping near the RGD site on fibronectin (a generous gift from K. Yamada, NIH) that cross-reacts with chick fibronectin isoforms (White and Bennett, unpublished observations); and our exon IIIB antibodies specific for the region encoded by the alternatively spliced exon IIIB (White *et al.*, 1996). The extent of chondrogenesis in the cultures was assessed 24, 48, and 72 hr after plating as described above for high-density mesenchymal micromass cultures.

In some experiments, the micromass cultures treated with exon IIIA antibody were rinsed and fixed 24 and 48 hr after plating as described for immunohistochemistry. The location of exon IIIA antibody binding was then detected using biotin-conjugated secondary anti-mouse antibodies and the Histostain-SP kit (Zymed Laboratories).

### Peptide Inhibition of Chondrogenesis in Vitro

Fusion proteins corresponding to the regions encoded by exon IIIA and exon IIIB with maltose binding protein (MBP) were synthesized in *Escherichia coli* and purified from the resulting extracts as described previously (White *et al.*, 1996). The purified exon IIIA/MBP and exon IIIB/MBP fusion proteins will hereafter be referred to as Peptide A/MBP and Peptide B/MBP, respectively. Peptide A/MBP and Peptide B/MBP were individually added to high-density mesenchymal micromass cultures in concentrations ranging from 0.3 to 2.4  $\mu\text{g}/\text{ml}$  in Ham's F12 containing 10% FBS 2 hr after initial plating of the cells. The extent of chondrogenesis in the cultures was assessed 72 hr after plating as described above for high-density mesenchymal micromass cultures.

### Histochemical Staining of Peanut Agglutinin-Binding Cells in Micromass Cultures

Limb mesenchymal cells were plated in high-density micromass culture as described above and stained with the lectin, peanut agglutinin (PNA), to visualize cellular condensations at 24, 48, and 72 hr after initial cell plating as described previously (Zimmermann and Thies, 1984; Stringa and Tuan, 1996). Specifically, the cultures were rinsed with PBS, fixed with buffered 4% paraformaldehyde, rinsed again with PBS, and stained with 100  $\mu\text{g}/\text{ml}$  peroxidase-conjugated PNA (Sigma) for 30 min, washed in PBS, and developed using a Histostain-SP kit (Zymed Laboratories Inc.). The stained cultures were viewed and photographed using an Olympus IMT-2 microscope equipped with Hoffman optics.

### Immunoblots

High-density mesenchymal micromass cultures at 0, 24, 48, and 72 hr after cell plating were extracted with 4 M urea in 0.05 M phosphate buffer, pH 7.3, and passed through an 18-gauge needle. Protease inhibitors (4 mM phenylmethanesulfonyl fluoride, 2 mM N-ethylmaleimide, 2 mM iodoacetic acid, 100 mM leupeptin, 0.2 IU/ml aprotinin, and 4 mM EDTA, final concentrations) were immediately added to each extract. The extracts were continuously dialyzed against PBS overnight using Slide-A-Lyzer 10K dialysis cassettes (Pierce Chemical Co., Rockford, IL) and proteins were

determined by the Bradford method using a Coomassie Plus kit (Pierce Chemical Co.). Equal amounts of protein (10  $\mu$ g) from each extract were then subjected to SDS-polyacrylamide gel electrophoresis on an 8% polyacrylamide minigel with a 4% stacking gel according to the procedure of Laemmli (1970). The proteins in the gels were transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) using a Mini-Protean II transfer apparatus (Bio-Rad Laboratories) in 25 mM Tris base, 190 mM glycine, 20% methanol as suggested by the manufacturer. Following transfer, individual, identical blots were processed with the general fibronectin antibodies (1:2000 dilution), the exon IIIA antibody (1:2000 dilution), or the exon IIIB antibodies (1:500 dilution) exactly as described and characterized previously (White *et al.*, 1996).

### **Shell-less Chick Embryo Culture**

For *in vivo* perturbation of limb chondrogenesis with fibronectin antibodies, shell-less chick embryos were maintained in shell-less culture (Tuan, 1980; Oberlender and Tuan, 1994a). Briefly, after 3 days of incubation *in ovo*, chick embryos were placed without the eggshell into ringstands lined with plastic kitchen wrap, covered with petri dish lids, and maintained in a humidified chamber at 37.5°C with constant air flow. This method permitted easy access to the limb and continuous observation of embryonic development.

### **In Vivo Injection of Chick Limb Primordia with Fibronectin Antibodies**

The antibodies were injected into the right limb primordia of HH stage 23/24 chick embryos in shell-less culture using a pulled 10- $\mu$ l capillary tube attached to a Hamilton microsyringe mounted on a micromanipulator (Oberlender and Tuan, 1994a). The volume injected was typically 0.5 to 1.0  $\mu$ l (5–10  $\mu$ g). The antibodies were suspended in Nile blue sulfate to visualize the efficacy of each injection. Injected limbs were assessed for chondrogenesis by whole-mount Alcian blue staining as described below.

### **Whole-Mount Alcian Blue Staining of Chick Embryos**

Two days after injection, the embryos were removed from the shell-less culture, fixed in Carnoy's solution, and subjected to whole-mount Alcian blue staining (Kimmel and Trammell, 1981; Oberlender and Tuan, 1994a). Briefly, the embryos were stained in acid-alcohol Alcian blue for 17 hr, cleared, and macerated in 2% KOH, hardened in 50% glycerol, and stored in 100% glycerol. The limbs were viewed and photographed using a Wild stereo microscope. All comparisons were made to the contralateral uninjected limb as well as to the same limb from another embryo injected with nonimmune mouse IgG.

## **RESULTS**

### **Immunohistochemical Localization of Fibronectin Isoforms in Chick Limb Buds *in Situ* and in High-Density Mesenchymal Micromass Cultures *in Vitro***

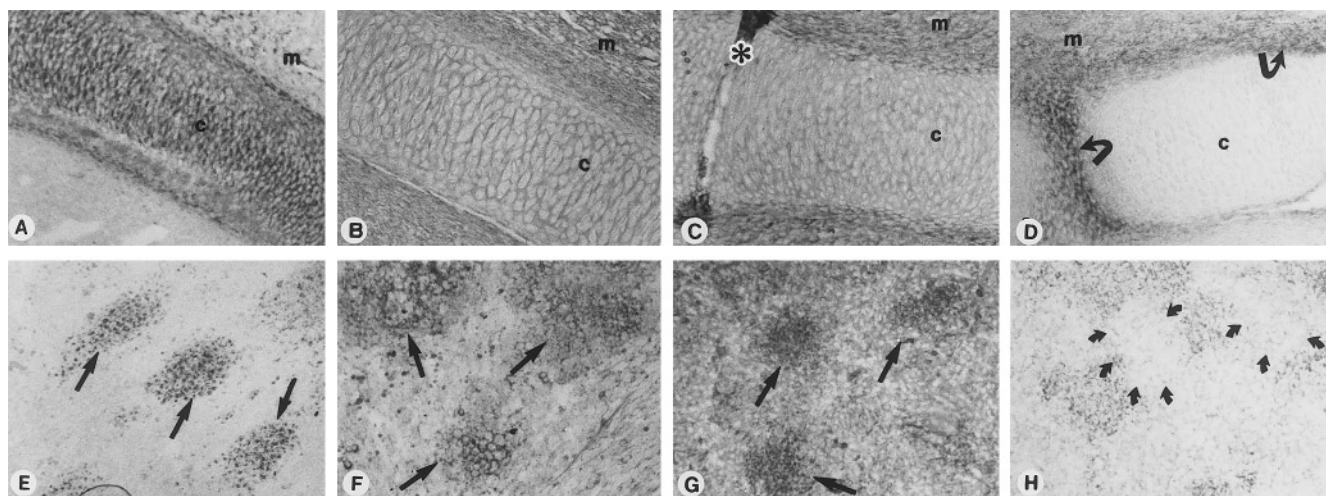
The developing chick limb provides a well-characterized system for the study of chondrogenesis *in vivo* (Thorogood and Hinchliffe, 1975; Ede, 1983; Newman *et al.*, 1985). Fur-

thermore, limb mesenchymal cells plated in high-density micromass cultures proceed through the condensation and differentiation stages of chondrogenesis over the course of 2–3 days according to the same sequence as that of the limb bud *in vivo* (Ahrens *et al.*, 1977; San Antonio and Tuan, 1986). Therefore, the developing chick limb and high-density mesenchymal micromass cultures represent ideal systems for examining the relationships between fibronectin mRNA alternative splicing and chondrogenesis.

Chick limb buds at various stages of development were examined by immunohistochemistry for the presence of each fibronectin isoform utilizing exon-specific antibodies for fibronectin. In HH stage 30 limbs, intense collagen type II staining is associated with cartilaginous regions (Fig. 1A) as previously reported (von der Mark, 1980) and clearly distinguishes cartilage and mesenchymal tissues. Fibronectin staining is also consistent with previously reported localization of fibronectin in the developing chick limb (Tomasek *et al.*, 1982). Specifically, staining with antibodies that recognize all fibronectin isoforms (FN-gen) reveals relatively homogeneous staining in the mesenchyme of early HH stage 20 limbs (not shown), highly localized staining in regions of condensation in HH stage 24 limbs (not shown), and then general staining in both the cartilage and the surrounding mesenchyme in the HH stage 30 limbs (Fig. 1B).

Immunostaining for the regions encoded by exon IIIA and exon IIIB is present as early as HH stage 19/20, shortly after limb bud formation, and exhibits a homogeneous staining throughout the limb mesenchyme (not shown). Similarly, in HH stage 23/24 limbs, fibronectin protein containing exon IIIA and IIIB is distributed throughout the limb (not shown). In contrast, the fibronectin protein containing the region encoded by exon IIIA (FN-A) disappears from cartilaginous regions of late HH stage 30 limbs (Fig. 1D), while fibronectin protein containing the region encoded by exon IIIB (FN-B) remains throughout the cartilage and surrounding mesenchyme (Fig. 1C). These results suggest that the fibronectin protein in prechondrogenic mesenchyme is B+A+ (mesenchymal fibronectin), while the fibronectin in limb cartilage is B+A- (cartilage fibronectin), in agreement with the fibronectin mRNA splicing patterns in these tissues (French-Constant and Hynes, 1989; Bennett *et al.*, 1991; Gehris *et al.*, 1996) and the characterization of fibronectin isoforms during chick (White *et al.*, 1996) and mouse (Peters *et al.*, 1996a,b) development.

In micromass cultures *in vitro*, mesenchymal cells aggregate to form discrete chondrogenic nodules, characterized by collagen type II immunostaining (Fig. 1E), separated by fibroblasts and myocytes (Ahrens *et al.*, 1977; San Antonio and Tuan, 1986). At 24 hr after plating, all of the fibronectin proteins are expressed at low, but detectable, amounts in both the aggregating mesenchyme and the loose mesenchyme in the surrounding areas. The staining for both FN-gen (Fig. 1F) and FN-B (Fig. 1G) in the condensing areas increases as a function of time. These proteins appear throughout the culture but are most highly expressed within the nodular areas. On the other hand, staining for FN-A increases in the aggregates until about 48 hr after



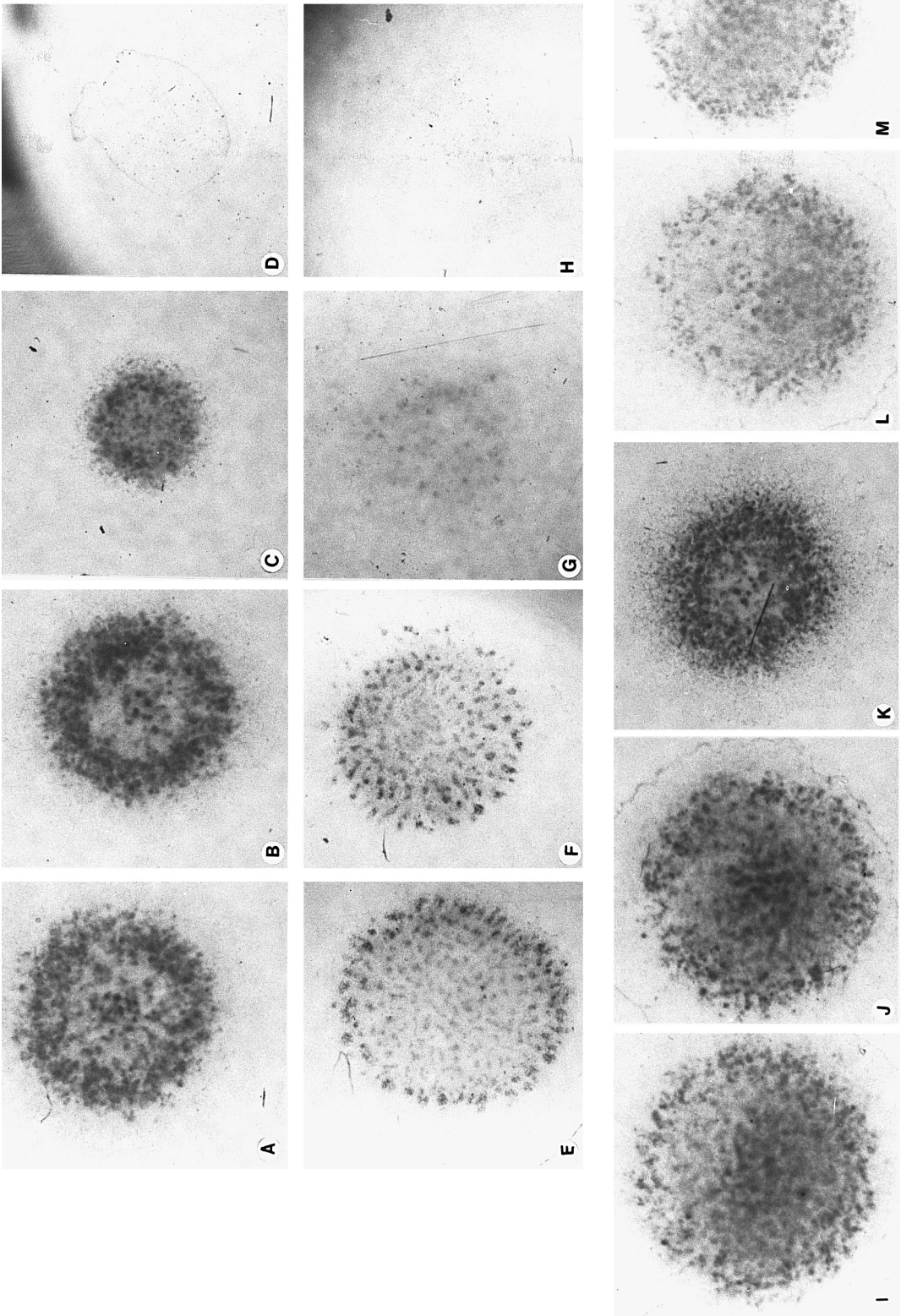
**FIG. 1.** Immunohistochemical localization of fibronectin isoforms in the chick limb bud from HH stage 29/30 embryos and high-density mesenchymal micromass cultures 72 hr after plating. Four closely adjacent longitudinal sections of the chick limb bud from HH stage 29/30 embryos were stained with an antibody specific for collagen type II (A), the general fibronectin antibodies (B), the exon IIIB antibodies (C), or the exon IIIA antibody (D) and viewed with bright-field optics. Staining for collagen type II demarcates the limb cartilage (c). Prominent staining with all three fibronectin antibodies (B–D) was seen in the perichondrium (curved arrows) and the adjacent condensing mesenchyme (m) surrounding the limb cartilage (c). Staining for general fibronectin (B) and for fibronectin containing the region encoded by exon IIIB (C) remains present in the differentiated cartilage, while fibronectin containing the region encoded by exon IIIA disappears from the limb cartilage. A fold in the tissue section is designated by the asterisk (\*). Original magnification is at 50 $\times$ . High-density mesenchymal micromass cultures were fixed for whole-mount immunohistochemistry and stained with an antibody to collagen type II (E), the general fibronectin antibodies (F), the exon IIIB antibodies (G), and the exon IIIA antibody (H) 72 hr after plating as described under Materials and Methods. Cellular aggregation and cartilage nodule formation are indicated by arrows (E–H) and collagen type II staining (E). Staining for general fibronectin (F) and FN-B is present within the nodules as well as in the internodular spaces (G). In contrast, FN-A staining is present only in the internodular spaces; staining has disappeared from the nodular areas (H). Original magnification is at 50 $\times$ .

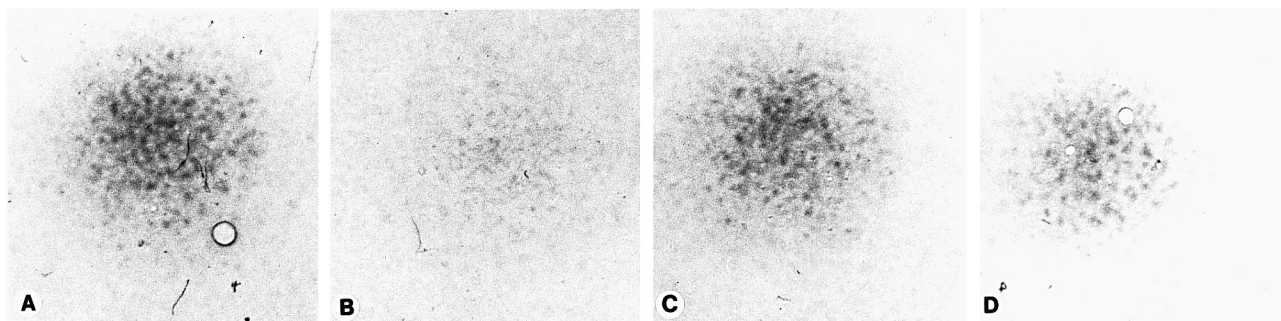
plating. The FN-A staining then disappears from the nodular areas while the surrounding mesenchyme continues to express the protein (Fig. 1H). Therefore, the temporal and spatial localization of fibronectin protein containing the region encoded by exon IIIA (FN-A) disappears just after condensation, in agreement with the temporal and spatial localization of the FN-A mRNA splicing patterns during chondrogenesis *in vitro* (Gehris *et al.*, 1996). The combination of these results implies that the mesenchymal fibronectin isoform (B+A+) is replaced with the cartilage fibronectin isoform (B+A–) as a result of a change in fibronectin mRNA alternative splicing just after the condensation event that occurs during chondrogenesis *in vitro* exactly as it occurs *in vivo*.

#### **Antibodies and Peptides Specific for the Region Encoded by Exon IIIA in Mesenchymal Fibronectin Inhibit Chondrogenesis *In Vitro***

To assess the functional role of fibronectin isoforms during chondrogenesis, we perturbed fibronectin activity in the high-density mesenchymal micromass cultures using exon-specific antibodies. Specifically, various fibronectin antibodies were added at increasing concentrations to the culture medium of limb mesenchymal cells plated in micro-

mass cultures 2 hr after initial cell plating. Three days later, treated and nontreated cultures were assessed for chondrogenesis by Alcian blue staining and *in situ* hybridization for collagen type II mRNA. Increasing concentrations of the exon IIIA antibody from 0.1 to 1.0  $\mu\text{g/ml}$  decreases the number of chondrogenic nodules formed in the cultures as viewed by Alcian blue staining (Figs. 2A–2D) and *in situ* hybridization for collagen type II mRNA (Figs. 2E–2H). The *in situ* hybridization results suggest that the presence of the antibody does not only inhibit Alcian blue binding to aggrecan glycosaminoglycans, but instead reflects an actual reduction in cartilage formation. Note that the area of the cultures containing chondrogenic nodules decreases in size with increasing amounts of antibody, suggesting that the antibody blocks cartilage formation most effectively in the less mature areas of the cultures (outermost regions of the micromass). Lot to lot variability of the exon IIIA antibody often results in variable inhibition of chondrogenesis (compare Fig. 2D with Fig. 4A). However, daily additions of the antibody from any lot (not shown) augments the inhibitory effect, suggesting that antibody binding to newly synthesized mesenchymal fibronectin is necessary to completely block chondrogenesis. In contrast, the addition of nonimmune rabbit IgG antibodies demonstrates no change in the number of chondrogenic nodules (Fig. 2I), regardless of antibody concentration compared to untreated control (Fig. 2A).





**FIG. 3.** Peptide inhibition of chondrogenesis *in vitro*. High-density mesenchymal micromass cultures were treated with 2.4  $\mu\text{g}/\text{ml}$  of Peptide A/MBP, Peptide B/MBP, and MBP (see Materials and Methods) after 2 hr in culture (B–D, respectively) and then stained with Alcian blue 72 hr after plating. Only Peptide A/MBP (B) inhibits chondrogenesis compared to untreated control (A). Original magnification is at 25 $\times$ .

To confirm that the inhibition of chondrogenesis by the addition of the exon IIIA antibody is a specific effect of the exon IIIA antibody rather than nonspecific effects of any antibody directed against fibronectin, we completed identical experiments with several control antibodies. Each preparation of antibodies was added at 1  $\mu\text{g}/\text{ml}$  to the cultures 2 hr after plating; a description of each antibody appears under Materials and Methods. The addition of affinity-purified exon IIIB antibodies (Fig. 2J), the 16G3 monoclonal antibody directed toward an epitope of fibronectin located close to the RGD site in the type III-10 repeat (Fig. 2L), and the B3D6 monoclonal antibody directed toward an unmapped epitope of chick fibronectin (Fig. 2M) had no effect on chondrogenesis suggesting at least a partial specificity for the region encoded by exon IIIA in the chondrogenic process. The general fibronectin antibodies (prepared against human plasma fibronectin which does not contain the regions encoded by either exon IIIA or exon IIIB) had only a modest effect on chondrogenesis (Fig. 2K), consistent with previous reports (Frenz *et al.*, 1989a,b) that other regions of the fibronectin molecule, such as the heparin-binding domain in the amino-terminal portion of the fibronectin molecule (Frenz *et al.*, 1989a,b), are also involved in the chondrogenic process. The combination of these results suggests that the region encoded by exon IIIA in mesenchymal fibronectin, acting possibly with one or more other domains in the fibronectin molecule, is specifically required for chondrogenesis.

The addition of increasing concentrations of Peptide A/MBP (Fig. 3B) but not Peptide B/MBP (Fig. 3C) or MBP (Fig.

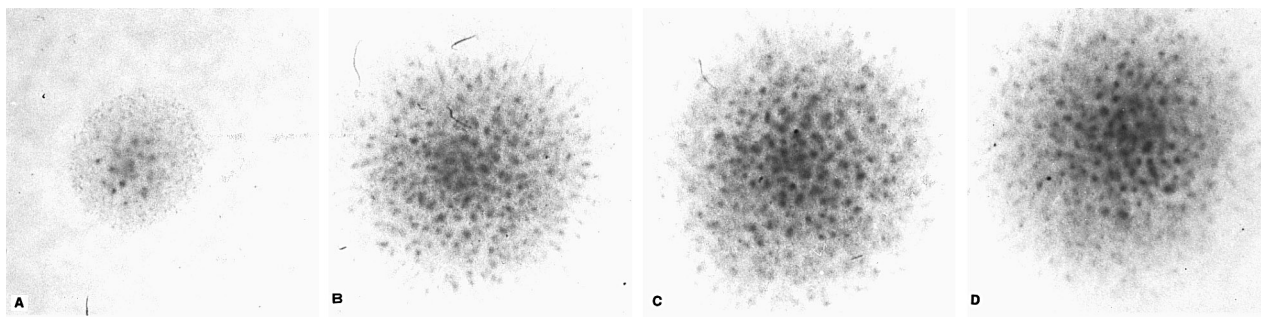
3D) (see Materials and Methods) to the micromass cultures 2 hr after plating also inhibits chondrogenesis in a dose-dependent manner with higher concentrations of the peptide having a greater effect on chondrogenesis. These results indicate that Peptide A/MBP specifically competes for binding of some unknown molecule(s) to the region encoded by exon IIIA present in mesenchymal fibronectin to exert its inhibitory effect on chondrogenesis.

### ***The Exon IIIA Antibody Inhibits Formation of Condensations during Chondrogenesis***

The down-regulation of both the FN-A mRNA (Gehris *et al.*, 1996) and protein (White *et al.*, 1996; Peters *et al.*, 1996a,b; Fig. 1) just after condensation *in vivo* and *in vitro* suggests that the inhibition of chondrogenesis resulting from the addition of the exon IIIA antibody prior to condensation might actually be the result of the antibody preventing formation of the condensations themselves. Cellular condensation *in vitro* begins in the center of the micromass cultures at about 18 hr after initial cell plating and then expands outward toward the periphery so that condensation is essentially complete in the outermost regions by about 48–72 hr after plating (San Antonio and Tuan, 1986). Therefore, we performed two additional experiments to examine more directly the effect of exon IIIA antibody addition on the formation of condensations. First, an experiment addressing the time-dependence of antibody addition on the inhibition of chondrogenesis revealed a correlation between

**FIG. 2.** Dose-dependent exon IIIA antibody inhibition of chondrogenesis *in vitro*. High-density mesenchymal micromass cultures were treated with increasing amounts (0.125, 0.25, 0.5, and 1  $\mu\text{g}/\text{ml}$ ) of exon IIIA antibody 2 hr after plating and stained for the presence of sulfated proteoglycans by Alcian blue (A–D, respectively) and then subjected to *in situ* hybridization for collagen type II mRNA (E–H, respectively) 72 hr after plating. Note the dose-dependent inhibition of chondrogenesis in these cultures with increasing concentrations of the exon IIIA antibody. I–M illustrate micromass cultures treated with 1  $\mu\text{g}/\text{ml}$  mouse IgG (I), the exon IIIB antibodies (J), the general fibronectin antibodies (K), the 16G3 monoclonal antibody specific for an epitope mapping near the RGD site on fibronectin (L), and the B3D6 avian-specific anti-fibronectin monoclonal antibody with an unmapped epitope (M) 2 hr after initial cell plating and then stained with Alcian blue 72 hr after plating. Note that only the general fibronectin antibodies (K) had any observable effect on the area of the culture undergoing chondrogenesis compared to untreated controls within each individual experiment (example shown in L); the intensity of Alcian blue staining often varies from one experiment to another. Original magnification is at 25 $\times$ .



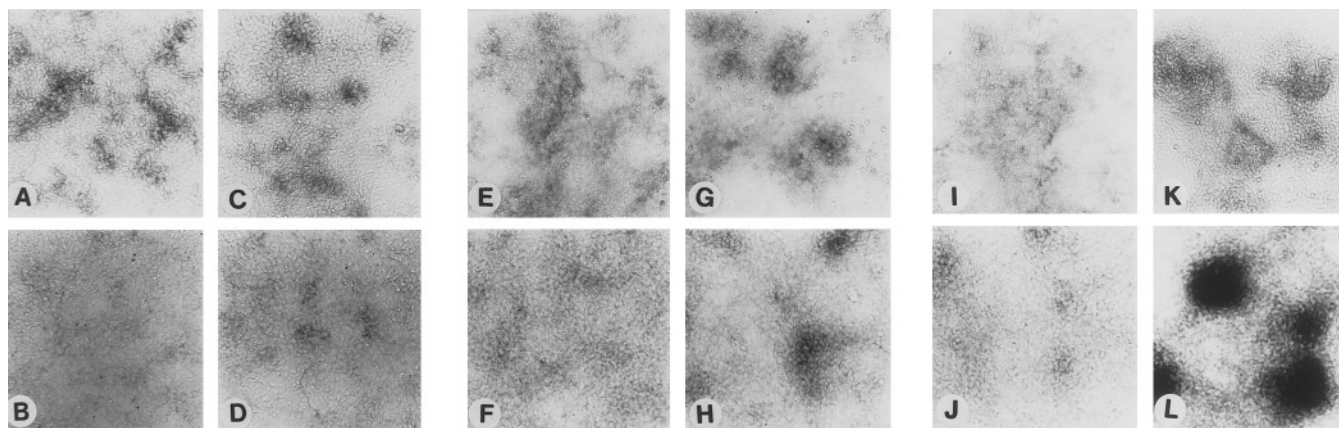


**FIG. 4.** Time-dependent exon IIIA antibody inhibition of chondrogenesis *in vitro*. High-density mesenchymal micromass cultures were treated with 1  $\mu\text{g}/\text{ml}$  of the exon IIIA antibody after 2, 18, and 42 hr in culture (A–C, respectively) or no antibody (D) and then stained with Alcian blue 72 hr after plating. Original magnification is at 25 $\times$ .

the time of antibody addition and the area of the micromass culture that undergoes chondrogenesis. Specifically, the addition of the exon IIIA antibody at earlier times after plating decreases the area of the culture that forms nodules (compare 2 hr versus 18 hr after plating in Figs. 4A and 4B, respectively). Furthermore, antibody addition following condensation (42 hr after plating in Fig. 4C) has essentially no effect on nodule formation (compare to no treatment in Fig. 4D). Together, these results suggest that the antibody may inhibit nodule formation by binding fibronectin present in regions of mesenchymal cells undergoing active condensation and thus preventing further formation of the condensations in the expanding cultures.

The effect of the exon IIIA antibody on cellular condensation was further assessed by staining with PNA, a galactose-specific lectin, as described under Materials and Methods. PNA binds specifically to the surfaces of presumptive chon-

drocyte precursors in the precartilage condensations prior to deposition of cartilage-specific extracellular matrix materials (Zimmermann and Thies, 1984; Aulthouse and Solursh, 1987; Milaire, 1991). Because PNA staining is thought to demarcate cellular condensations during the development of skeletal tissues, this method provides a visual assessment of condensation formation in the cultures (Hall and Miyake, 1992,1995). Micromass cultures were treated with 1  $\mu\text{g}/\text{ml}$  exon IIIA antibody (Fig. 5, left half of each panel) or no antibody (Fig. 5, right half of each panel) 2 hr after plating. Antibody-treated and untreated cultures were then assessed for cellular condensation by PNA staining (Fig. 5, top row) and for chondrogenesis by Alcian blue staining of the cartilaginous nodules (Fig. 5, bottom row) 24 hr (Fig. 5, left panel), 48 hr (Fig. 5, middle panel), and 72 hr (Fig. 5, right panel) after plating. PNA staining of condensations in the center of untreated cultures appears organized



**FIG. 5.** Localization of precartilage condensations in micromass cultures by PNA binding. High-density mesenchymal micromass cultures were incubated in the presence (left half of each panel) and absence (right half of each panel) of 1  $\mu\text{g}/\text{ml}$  exon IIIA antibody added 2 hr after cell plating and then stained with PNA-peroxidase (top row) as described under Materials and Methods or Alcian blue (bottom row) 24 hr (left panel), 48 hr (middle panel), and 72 hr (right panel) after plating. The addition of the exon IIIA antibody inhibits formation of cartilaginous nodules exhibited by Alcian blue staining (compare bottom left with bottom right in each panel) and disrupts condensation formation as exhibited by PNA staining (compare top left with top right in each panel) with time in culture. Original magnification is at 50 $\times$ .



by 24 hr (Fig. 5C) and continues to accumulate in the center (Figs. 5G and 5K) and expand outward toward the periphery of the cultures with time. Alcian blue staining of parallel untreated cultures demonstrates the normal progressive formation of cartilaginous nodules over the same 3-day culture period (Fig. 5, bottom right of each panel).

The addition of the exon IIIA antibody to the high-density micromass cultures 2 hr after plating alters both the PNA and Alcian blue staining patterns over the course of the culture period. Specifically, the formation of cartilaginous nodules is dramatically inhibited in antibody-treated cultures compared to the untreated control cultures at each time point (Fig. 5, compare cultures treated with antibody in the bottom left corner with untreated cultures in the bottom right corner of each panel), in agreement with results shown in Fig. 2. Note that the sizes of individual cartilaginous nodules are substantially smaller in the treated cultures than in untreated cultures (compare Fig. 5J to 5L), suggesting that the exon IIIA antibody inhibits the size as well as the number (see Fig. 2) of chondrogenic nodules in the cultures. The PNA staining of the antibody-treated cultures, however, indicates that condensation initiation occurs equally well in the center of the cultures after 24 hr (compare Fig. 5A with 5C), but then the central condensations appear to become progressively disrupted after 48 and 72 hr (compare Figs. 5E and 5G with 5I and 5K, respectively), while peripheral condensations never initiate. Thus, the exon IIIA antibody appears to affect formation and maintenance of precartilage condensations.

Because the micromass cultures consist of cells enzymatically dissociated from the limb buds, we examined whether the absence of exon IIIA antibody interference with initial mesenchymal cell aggregation after 24 hr of micromass culture (Fig. 5A) might be related to the resynthesis and matrix deposition of mesenchymal fibronectin during this initial phase of culture. Specifically, we performed three different experiments as a means to compare the amount of mesenchymal fibronectin present in the matrix at 24 and 48 hr. First, we localized exactly where the exon IIIA antibody binds within the treated cultures at 24 and 48 hr using a biotinylated goat anti-mouse IgG secondary antibody (Fig. 6A). The results showed very little, if any, staining above background after 24 hr of culture (compare Fig. 6A with Fig. 6B), compared to the readily detectable signal in the matrix at 48 hr (compare Fig. 6C with Fig. 6D). Second, we immunofluorescently colocalized fibronectin containing the regions encoded by exon IIIA (rhodamine label) and exon IIIB (fluorescent label) at 24 and 48 hr after plating (Fig. 6B). At both time points, the signals for the regions encoded by exon IIIA and exon IIIB codistribute, indicating that the fibronectin present in the cultures is actually mesenchymal fibronectin and not an isoform of fibronectin that does not contain exon IIIA. In addition, more mesenchymal fibronectin is clearly present in the matrix of the cultures after 48 hr than after 24 hr (compare Figs. 6E and 6F with 6G and 6H). Finally, Western immunoblot analysis of 4 M urea extracts from the micromass cultures at 0, 24, 48, and 72 hr with the general, exon IIIA, and exon IIIB antibodies shows that the amounts

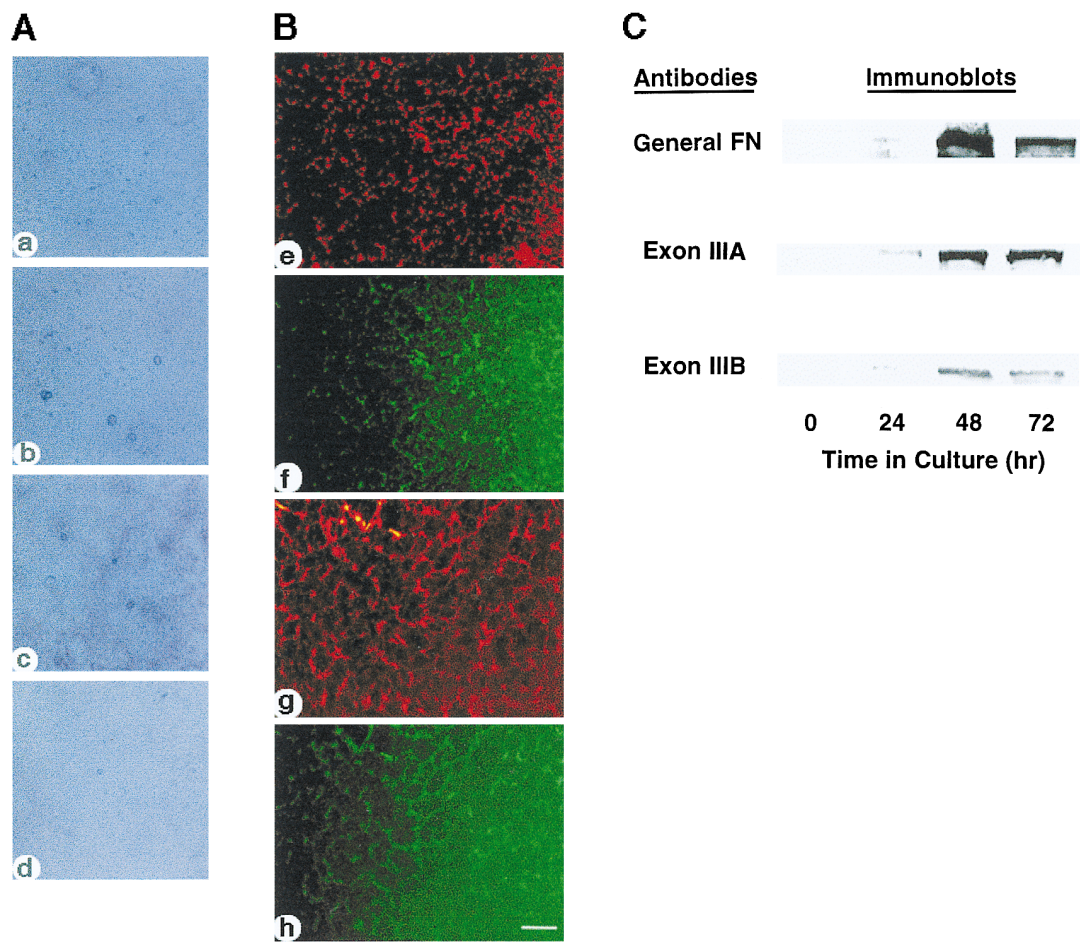
of fibronectin increase with time in culture and the amount of mesenchymal fibronectin present in the 48-hr extracts is significantly greater than the amount in the 24-hr extracts. The combination of these results suggests that the exon IIIA antibody may not inhibit condensation initiation in the center of the cultures, visualized by PNA staining (compare Figs. 5A and 5C), most likely because of insufficient amounts of fibronectin that has been resynthesized and deposited into the matrix by 24 hr after plating. In any event, the results of the PNA staining, combined with the results of the temporal studies of exon IIIA antibody addition, suggest that the inhibition of chondrogenesis in the presence of the exon IIIA antibody occurs during the condensation event. Thus, the region encoded by exon IIIA in mesenchymal fibronectin appears to be involved in cellular condensation that occurs during chondrogenesis.

### **The Exon IIIA Antibody Perturbs Chondrogenesis in the Developing Chick Embryonic Limb**

Since the exon IIIA antibody inhibits chondrogenesis *in vitro* (Fig. 2), we wanted to determine whether perturbation of mesenchymal fibronectin function with the exon IIIA antibody would also inhibit chondrogenesis *in vivo*. The exon IIIA antibody was injected into the limbs of shell-less chick embryos (Tuan, 1980) at HH stage 22–23, corresponding to the time of active cellular condensation and when the fibronectin present in the mesenchyme contains the region encoded by exon IIIA. Interestingly, injection of the exon IIIA antibody appears to disrupt normal limb chondrogenesis resulting in moderate to severe skeletal malformations 2 days after exon IIIA antibody injection into the fore- and hindlimbs of the embryos (Fig. 7). In one representative experiment, 10 of the 12 embryos injected with the exon IIIA antibody (83%) exhibited some degree of limb deformity. In contrast, none of the embryos injected with the exon IIIB antibodies (five embryos), mouse IgG (five embryos), or dye alone (five embryos) exhibited any malformations. Thus, the malformations do not appear to be the result of the injection procedure or blocking of the region encoded by the alternatively spliced exon IIIB in mesenchymal fibronectin.

Several of the limbs injected with the exon IIIA antibody appear small (Figs. 7B, 7C, and 7F), while others are more misshapened (Figs. 7D and 7E). Alcian blue staining of the limbs injected with the exon IIIA antibody and their respective contralateral control limbs (Figs. 7D–7F) reveals the absence of one or more skeletal elements in the antibody-injected limbs. For example, the injected limb in Fig. 7D has a shorter fibula and tibia, an underdeveloped fibulare, a shortened humerus, and a missing digit (see Figs. 7E and 7F for additional examples). The limbs injected with equivalent concentrations of nonimmune rabbit IgG (Fig. 7I), the exon IIIB antibodies (Fig. 7G), or dye alone (Fig. 7H) did not show any changes in morphology or Alcian blue staining compared to contralateral uninjected control limbs.

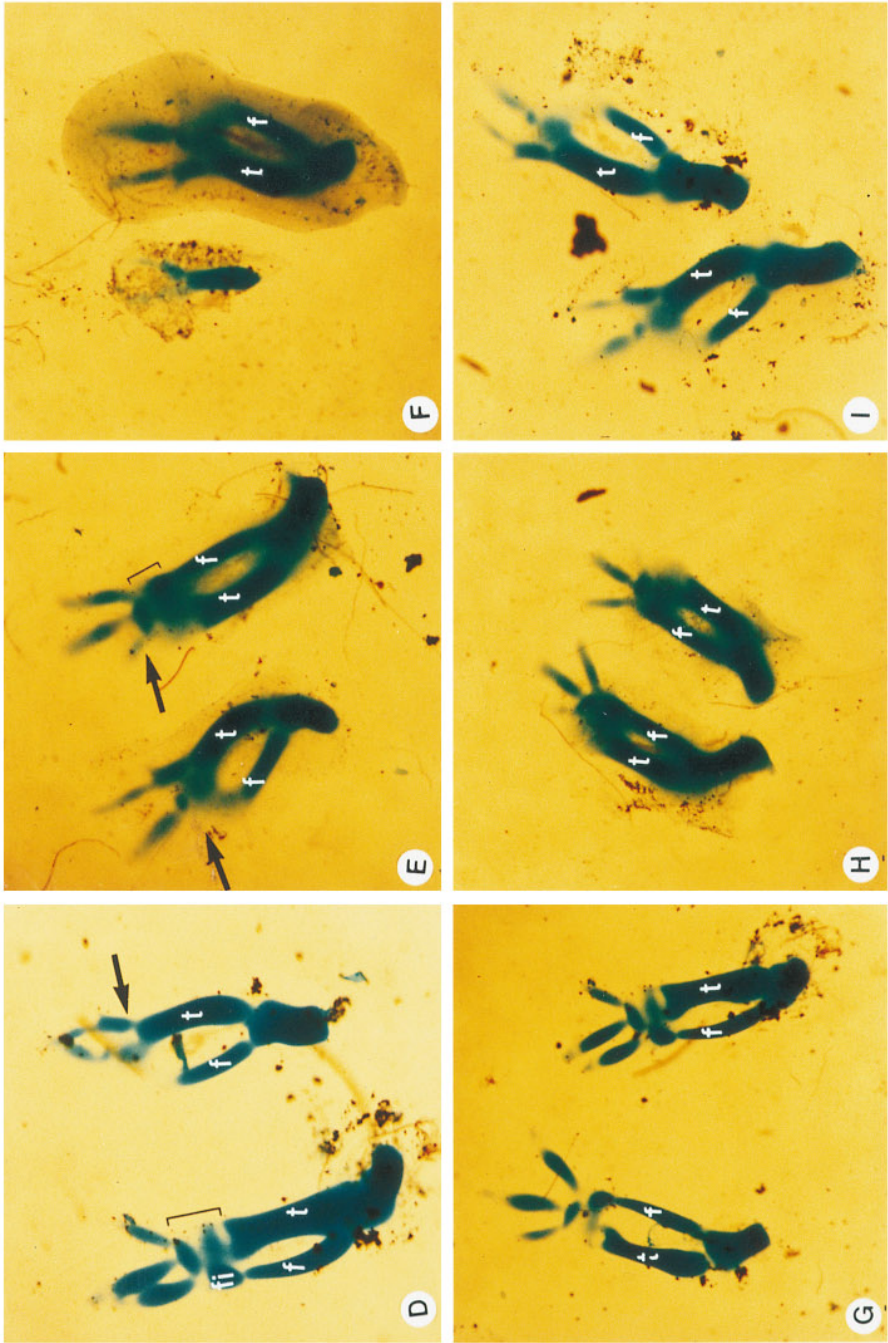
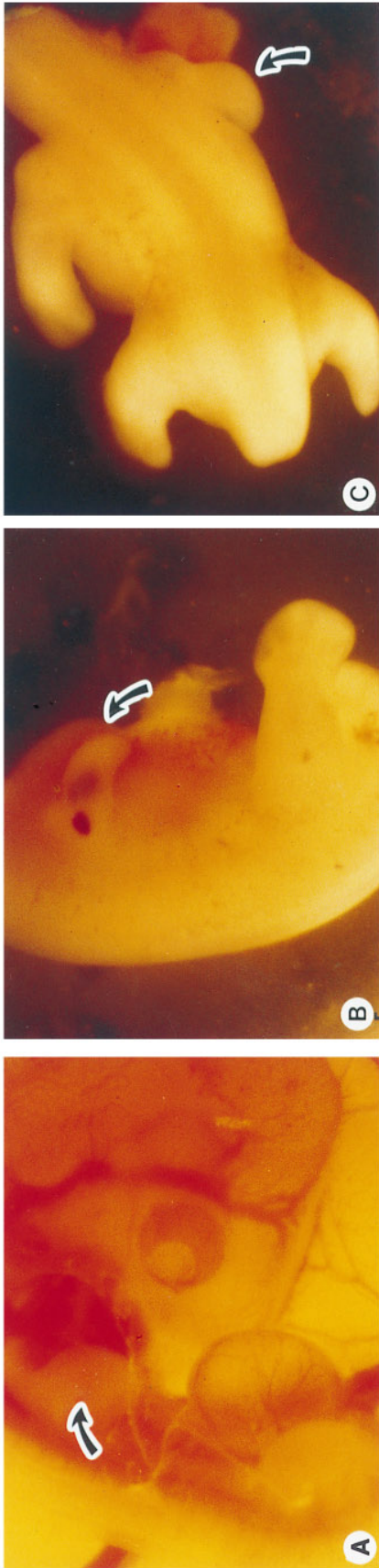
The combination of the *in vitro* and *in vivo* results strongly suggests that the region encoded by exon IIIA pres-



**FIG. 6.** Localization and levels of mesenchymal fibronectin in mesenchymal micromass cultures. (A) Two hours after initial cell plating, high-density mesenchymal micromass cultures were incubated in the presence (a and c) and absence (b and d) of exon IIIA antibody. The exon IIIA antibody-treated and untreated (no first antibody background controls) cultures were then rinsed and fixed for immunohistochemistry as described under Materials and Methods after 24 hr (a, b) or 48 hr (c, d) in culture. The binding locations of the exon IIIA antibody in the treated and untreated cultures were then visualized with a biotin-conjugated secondary anti-mouse antibody (a–d) or no secondary antibody (not shown) and the Histostain-SP kit as described under Materials and Methods. Original magnification is at 50 $\times$ . (B) Mesenchymal cells were plated at lower cell density to facilitate visualization by fluorescence as described under Materials and Methods. After 24 hr (e and f) and 48 hr (g and h), cultures were incubated simultaneously with both the exon IIIA and exon IIIB antibodies. The exon IIIA antibody was then visualized with a rhodamine-conjugated goat anti-mouse antibody (e and g), while the exon IIIB antibody was visualized with a fluorescein-conjugated goat anti-rabbit antibody (f and h) as described under Materials and Methods. Original magnification is at 50 $\times$ ; the bar represents 50  $\mu$ m. (C) Extracts were prepared from 0-, 24-, 48-, and 72-hr high-density mesenchymal micromass cultures as described under Materials and Methods. Equal amounts of protein (10  $\mu$ g/lane) from each extract were then separated on 8% polyacrylamide minigels and subjected to SDS–polyacrylamide gel electrophoresis. The separated proteins were then transferred to nitrocellulose and the immunoblots processed with the general fibronectin antibodies (top), the exon IIIA antibody (middle), and the exon IIIB antibodies (bottom) as described under Materials and Methods.

**FIG. 7.** Exon IIIA antibody inhibition of chondrogenesis *in vivo*. Limb primordia from HH stage 22–23 shell-less chick embryos were injected with 1  $\mu$ g exon IIIA antibody, cultured for an additional 2 days, and then subjected to whole-mount Alcian blue staining as described under Materials and Methods. (A) Uninjected embryo; arrow indicates antibody injection site in the hindlimb of an HH stage 23 embryo. (B) Embryo 2 days after exon IIIA antibody injection into the forelimb (arrow) compared to the hindlimb of the same embryo. (C) Embryo 2 days after exon IIIA antibody injection into the forelimb (arrow) compared to the contralateral uninjected forelimb of the same embryo. D–F illustrate examples of the effects of antibody injections on the morphology of the primitive hindlimb (D and E) and the forelimb (F) after Alcian blue staining compared to the respective contralateral uninjected limbs from the same embryos. In D, the injected limb (right) has a shorter fibula (f) and tibia (t), an underdeveloped fibulare (fi), a shortened humerus, and a missing digit (arrow). In E, the injected limb (left) is missing the fibulare, both the fibula and tibia are small, and one digit is missing. In F, the Alcian blue-stained forelimb from the embryo shown in C (bottom) exhibits gross skeletal deformity including a marked reduction in size of all the skeletal elements as well as the digits compared to the contralateral control (right). G–I illustrate limbs injected with the exon IIIB antibodies, dye alone, and mouse IgG, respectively. None of the injected limbs (left) show any malformations compared to the contralateral controls (right). f, fibula; fi, fibulare; t, tibia.





ent in mesenchymal fibronectin is necessary for chondrogenesis. Furthermore, the time dependency of exon IIIA antibody addition and the alteration in PNA staining following exon IIIA addition in the high-density mesenchymal micromass cultures suggest that this region in mesenchymal fibronectin is specifically required for some aspect of the condensation event that occurs during chondrogenesis.

## DISCUSSION

Alternative RNA splicing during developmental processes appears to provide a mechanism for the creation of protein heterogeneity. Structural diversity of fibronectin, resulting from the alternative splicing of exon IIIA, occurs during chondrogenesis, the differentiation of mesenchyme into cartilage (Bennett *et al.*, 1991). Specifically, the splicing patterns of fibronectin mRNAs change from B+A+V+ in mesenchyme to B+A-V+ in differentiated cartilage just after the condensation event that occurs during chondrogenesis (Gehris *et al.*, 1996). In this report, we have confirmed by immunohistochemical localization with exon-specific antibodies that the fibronectin isoforms at the protein level change during chondrogenesis in the developing chick limb *in vivo* and in high-density mesenchymal micromass cultures *in vitro* (Fig. 1), exactly as the splicing patterns of the fibronectin mRNAs change in these systems (Gehris *et al.*, 1996). Specifically, the region encoded by exon IIIA is present in fibronectin mRNAs prior to condensation (mesenchymal fibronectin) and then disappears just after condensation (cartilage fibronectin). Thus, the developmental timing of this change in fibronectin isoform structure during chondrogenesis (Fig. 1 and Gehris *et al.*, 1996), coupled with the increase in the expression of fibronectin coincident with cellular condensation (Searls *et al.*, 1973; Thorogood and Hinchliffe, 1975; Kulyk *et al.*, 1989), suggests that the presence of the region encoded by exon IIIA in mesenchymal fibronectin may be necessary for the condensation event itself and ultimately for the chondrogenic process.

We have tested this hypothesis by perturbing chondrogenesis *in vitro* and *in vivo* with exon-specific antibodies to fibronectin. The addition of the exon IIIA antibody inhibits chondrogenesis *in vitro* (Fig. 2) and *in vivo* (Fig. 7). The timing of the exon IIIA antibody addition also has an effect on chondrogenesis; addition of the exon IIIA antibody prior to condensation inhibits nodule formation in actively condensing areas (Fig. 3). Furthermore, exon IIIA antibody addition to high-density mesenchymal micromass cultures disrupts the pattern of PNA staining (Fig. 5), a visual assessment of condensations. Thus, the time dependency of exon IIIA antibody addition on chondrogenesis combined with the altered PNA staining in exon IIIA antibody-treated cultures supports our hypothesis that the region encoded by exon IIIA plays a role during the cellular condensation phase of chondrogenesis.

The precartilage condensation appears absolutely necessary for overt chondrogenesis since the extent of cellular

aggregation is directly correlated with the extent of cartilage nodule formation in mesenchymal cells undergoing chondrogenesis *in vitro* (Solursh, 1983). Other adhesion molecules besides fibronectin, such as *N*-cadherin (Oberlender and Tuan, 1994a,b) and N-CAM (Widelitz *et al.*, 1993), have also been implicated in this condensation process. However, because fibronectin primarily functions in matrix-matrix or cell-matrix interactions and *N*-cadherin and N-CAM primarily function in cell-cell interactions, they each probably contribute to different aspects of the condensation process. Since mesenchymal fibronectin is already present before the transient expression of *N*-cadherin and N-CAM *in vivo*, one might expect that fibronectin would be responsible for initially bringing the mesenchymal cells into close proximity to each other so that subsequent cell-cell interactions mediated by *N*-cadherin and N-CAM could be initiated for completion of the condensation event. However, PNA staining to visualize cellular condensations in the mesenchymal micromass cultures treated with the exon IIIA antibody suggests that cell aggregates form initially (while mesenchymal fibronectin levels are still very low following enzymatic dissociation of the limb buds) and then become disrupted (Fig. 5) as fibronectin levels increase (Fig. 6). These results are consistent with *N*-cadherin and/or N-CAM being responsible for the initial aggregation phase *in vitro* and then mesenchymal fibronectin playing a role in the secondary recruitment phase of condensation formation when additional mesenchymal cells are brought into the initial cell aggregates (Solursh *et al.*, 1982). The correlation between this *in vitro* scenario and the *in vivo* developmental program remains for further investigation.

Our immunohistochemical localization of fibronectin isoforms at different stages of chick limb development indicates that mesenchymal fibronectin (B+A+V+) is present throughout the early limb mesenchyme (not shown) and then increases dramatically in the areas of active condensation (Fig. 1), consistent with previous fibronectin immunolocalization studies (Searls, 1973; Thorogood and Hinchliffe, 1975; Dessau *et al.*, 1980; Melnick *et al.*, 1981; Kosher *et al.*, 1982; Tomasek *et al.*, 1982; Kulyk *et al.*, 1989). Because the structure of the fibronectin in the actively condensing mesenchyme is no different than the fibronectin in the noncondensing mesenchyme, the region encoded by exon IIIA obviously does not determine which mesenchymal cell subpopulation will form condensations. However, the levels of expression of mesenchymal fibronectin and/or expression of a specific receptor for mesenchymal fibronectin remain viable possibilities for these determining factors. The fact that the addition of Peptide A/MBP to high-density mesenchymal micromass cultures inhibits chondrogenesis (Fig. 3B) similar to the addition of exon IIIA antibody suggests that either another matrix component or a molecule on the mesenchymal cell surface binds directly to the region encoded by exon IIIA in mesenchymal fibronectin. The possibility that a specific receptor for this region may exist and serve not only as a marker for condensation, but also to facilitate the condensation process by interacting with the

region encoded by exon IIIA in mesenchymal fibronectin is an intriguing possibility.

Previous work has implicated fibronectin as playing a direct role in the formation of condensations during chondrogenesis (Newman *et al.*, 1985, 1987; Frenz *et al.*, 1987a,b; Leonard *et al.*, 1991). Most of this work has focused on an interaction of the N-terminal heparin-binding domain of fibronectin with heparin-like molecules on the mesenchymal cell surface. Our present work suggests that the region encoded by exon IIIA is also required for this condensation event and thus for subsequent chondrogenesis. The absence of an inhibition of chondrogenesis in the presence of monoclonal antibodies specific for a region adjacent to the RGD site (Fig. 2L), the region encoded by exon IIIB (Fig. 2J), and another unmapped epitope of the fibronectin molecule (Fig. 2M) provides specificity for the region encoded by exon IIIA in the mesenchymal fibronectin isoform. However, these experiments do not rule out the necessity of other regions, such as the heparin-binding domain, common to all fibronectin isoforms in the function of mesenchymal fibronectin during chondrogenesis.

The malformations obtained as a result of the injection of the exon IIIA antibody into the limb primordia of chick embryos in shell-less culture confirm that the region encoded by exon IIIA in mesenchymal fibronectin affects chondrogenesis *in vivo* as well as *in vitro*. Specifically, each limb malformation is characterized by either smaller or missing skeletal elements (Figs. 7D–7F). The obvious absence of supernumerary or duplication defects of the limbs suggests that the region encoded by exon IIIA in mesenchymal fibronectin does not exert its effect on chondrogenesis, and thus on skeletal formation, by providing patterning information for the limb as do agents such as retinoic acid (Tickle, 1991; Niederreither *et al.*, 1996) and fibroblast growth factor (Cohn *et al.*, 1995) or the expression of *sonic hedgehog* (Riddle *et al.*, 1993) or *hox* genes (reviewed in Tickle, 1995). Instead, fibronectin most likely serves as a “messenger” in the extracellular matrix for carrying out the information provided by these patterning genes and agents.

The experiments described in this report strongly suggest that the region encoded by exon IIIA in mesenchymal fibronectin (B+A+V+) is necessary for the formation and/or maintenance of mesenchymal cell condensations and thus for subsequent chondrogenesis. The functional significance of the absence of this region in cartilage fibronectin (B+A–V+) at later stages of chondrogenesis remains for future experiments. However, the functional necessity of the region encoded by exon IIIA in mesenchymal fibronectin for mesenchymal condensation demonstrated in this report strongly supports the idea that alternative splicing of fibronectin mRNAs during chondrogenesis provides a means of creating both structural and functional diversity in the fibronectin molecule while also maintaining fibronectin's overall function as a connecting molecule in the extracellular matrix.

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